JOURNAL OF VIROLOGY, May 1985, p. 525–531
0022-538X/85/050525-07$02.00/0

Vol. 54, No. 2

Sequence Organization and Molecular Cloning of Mouse Mammary Tumor Virus DNA Endogenous to C57BL/6 Mice

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Received 24 September 1984/Accepted 6 December 1984

The sequence organization of mouse mammary tumor virus DNA endogenous to the C57BL/6 inbred mouse strain was characterized by Southern blot analysis, utilizing probes specific for particular regions of the mouse mammary tumor virus provirus and by molecular cloning of endogenous mouse mammary tumor virus DNA. The genome of C57BL/6 mice contains three apparently intact, endogenous proviral units; two of these units comprise the *Mtv-8* (unit II) and *Mtv-9* (unit III) genetic loci that are also present in the DNA of BALB/c mice. The third unit is defined by *Eco*RI restriction fragments of 10.0 and 8.4 kilobases that contain the 5′ and 3′ portions of the provirus, respectively. This unit, termed unit XI and encoded by the genetic locus *Mtv-17*, has not been previously recognized in C57BL/6 DNA, but it can be clearly distinguished from the proviral units at *Mtv-8* and *Mtv-9* by Southern blot analysis under appropriate conditions. The proviral unit at *Mtv-17* is not present in BALB/c DNA. DNAs comprising the entire *Mtv-8* locus and the 3′ portions of *Mtv-9* and *Mtv-17* were cloned. Analysis of the cloned DNA revealed no obvious deletions or rearrangements that would render proviral DNA defective; however, these endogenous genes are normally not transcriptionally active.

The RNA genome of mouse mammary tumor virus (MMTV), a retrovirus, is encoded by proviral DNA covalently integrated into the nuclear DNA of infected cells. In cultured mouse mammary carcinoma cells or in many glucocorticoid receptor-bearing cell lines infected with MMTV in tissue culture, the rate of synthesis and steady-state concentration of MMTV RNA are rapidly and specifically increased by glucocorticoid hormones (26, 27, 34). In vitro, purified glucocorticoid receptor protein has been shown to bind specifically and with high affinity to a 340-base pair restriction fragment from the MMTV long terminal repeat (LTR) (11, 12, 22–24, 28). This fragment, termed the glucocorticoid response element, is able to confer hormone inducibility on heterologous promoters to which it is linked in *cis*, and many of its properties are similar to those of enhancer elements (5). The abundance of hormonally induced transcripts and, indeed, the ability of glucocorticoids to affect transcription at all appear to be a function of the chromosomal integration site of the provirus (10). Thus, there are apparently two levels at which viral transcription is regulated: the specific interaction of glucocorticoid receptor with regulatory DNA sequences and a much less clearly defined positional effect that may determine whether or not a productive interaction between receptor and DNA can occur.

MMTV proviral DNA is endogenous to all inbred strains of mice and to many feral populations. These endogenous sequences are stable genetic elements; their number and genomic location are a characteristic of each inbred strain (7, 33). Endogenous MMTV genes of many mouse strains are not actively transcribed. For example, the spleen, liver, or mammary gland of BALB/c mice contains no detectable MMTV RNA (19, 36) even though these mice contain three genomic regions with sequence homology to MMTV, two of which are apparently complete copies of proviral DNA (6). Similarly, C57BL/6 spleen and liver do not express endogenous MMTV genes, although some viral RNA is apparently synthesized in lactating mammary gland (19). In lactating mammary glands of the related strain C57BL, MMTV RNA is transcribed from endogenous proviral DNA, but expression of mature viral proteins is blocked at the level of translation (35).

The failure of an endogenous gene to respond to a hormonal stimulus may be due to defects inherent in the provirus itself (e.g., a defective glucocorticoid response element) or be related to its location in the genome (position effects). To begin to investigate these possibilities, we have examined the DNA sequence organization of endogenous MMTV genes in C57BL/6 cells by Southern blot analysis and molecular cloning. Our results have revealed several features of endogenous MMTV gene organization in these cells that have not been previously recognized.

MATERIALS AND METHODS

Cell lines. Continuous cell lines were lymphomas of T-cell origin. T1M1 is derived from the inbred mouse strain C57BL/6 (21, 31), VL3 is from C57BL/Ka (16), and W7 is from BALB/c (14). Cells were grown in suspension in Dulbecco modified Eagle medium (GIBCO Laboratories) containing 10% horse serum (KC Biologicals) in an atmosphere of 5% CO_2 saturated with water.

Preparation of DNA. For preparation of high-molecular-weight, genomic DNA, tissue culture cells were washed with isotonic saline and dissolved in a buffer containing 10 mM Tris-hydrochloride (pH 7.6), 0.1 M NaCl, 1 mM EDTA, 0.5% sodium dodecyl sulfate, and 50 μg of proteinase K (Bethesda Research Laboratories) per ml. After incubation at 37°C for 12 to 16 h, the solution was extracted twice with phenol-$CHCl_3$ (2:1) and once with $CHCl_3$. Traces of organic solvent were removed either by extensive dialysis against 10 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA or by two successive ethanol precipitations (the second from 2.5 M ammonium acetate).

Plasmid DNA was purified from chloramphenicol-treated *Escherichia coli* HB101 or TB1 by the alkaline extraction procedure of Birnboim and Doly (2) followed by banding in a CsCl-ethidium bromide density gradient.

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Restriction endonuclease digestion. Restriction endonucleases (Bethesda Research Laboratories or New England Biolabs) were used according to the supplier's recommendations. Complete digestion of mouse genomic DNA was monitored by an internal standard of DNA from bacteriophage λ.

Southern (29) blot analysis. A 10-μg portion of restriction endonuclease-digested DNA was fractionated by electrophoresis through a 1% agarose gel. The gel was treated with 0.5 M NaOH–1.5 M NaCl followed by 0.5 M Tris-hydrochloride (pH 7.6)–3 M NaCl (30 min each), and the DNA was blotted onto nitrocellulose (Millipore Corp. or Schleicher & Schuell). Filters were then air dried, baked for 2 to 3 h at 80°C in a vacuum oven, and hybridized as described below.

Molecular weight markers consisted of restriction fragments of cloned MMTV DNA whose sizes had been determined by comparison with *Hin*dIII-digested λ DNA or restriction fragments of pBR322 (3). The sizes of the markers are 14.1, 12.4, 10.7, 9.8, 8.0, 6.5, 6.0, 4.7, 4.4, 2.0, and 1.3 kilobases (kb).

Hybridization probes. Southern blots were hybridized with ^{32}P-labeled, recombinant plasmid DNA carrying one of the MMTV inserts shown in Fig. 1. Probes 2 and 3 are *Pst*I fragments contained in plasmids pMTV4 and pLTR1, respectively (34). Probes 1 and 6 are *Eco*RI-*Bgl*II and *Eco*RI-*Hin*dIII subclones of pMTV3 (18). These two fragments were inserted into the *Eco*RI-*Bam*HI and *Eco*RI-*Hin*dIII sites of pUC8 (37) to give plasmids pMTV3.3 and pMTV3.2, respectively. Probe 5 is pMTV1 (34). Probe 4 is a *Pst*I-*Eco*RI fragment from the 5′ portion of *Mtv-8* cloned in the present work. It was derived by partial digestion of the parental *Eco*RI fragment with *Pst*I and ligation into pUC8 to give the recombinant plasmid pTC8.

Radioactive labeling was by nick translation as described by Feinstein et al. (10) to a specific activity of 1×10^8 to 3×10^8 cpm/μg.

Filter hybridization conditions. Filters were prehybridized for 6 to 24 h at 41°C in sealed plastic bags containing 7 ml of a solution consisting of 3× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), 50% deionized formamide, 50 μg of denatured salmon sperm DNA per ml, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and 0.1% Ficoll per 100 cm^2. Prehybridization solution was removed and probe (0.5×10^6 to 1.0×10^6 cpm/ml) was added in the same solution containing 10% (wt/vol) dextran sulfate. After incubation at 41°C for 12 to 24 h, filters were washed with 0.1× SSC–0.1% sodium dodecyl sulfate several times at room temperature and then for 1 h at 50°C in several changes of the same solution. After drying, filters were exposed for 1 to 5 days to Kodak XAR-5 X-ray film with Dupont Cronex Lightning-Plus intensifying screens.

In some cases filters were hybridized sequentially with different DNA probes. This was accomplished by melting off the first probe by incubating the filter for 1 to 4 h at 68°C in prehybridization solution, removing the solution containing the melted probe, prehybridizing, and hybridizing with a new probe as described above.

Molecular cloning techniques. A 2-μg portion of T1M1 high-molecular-weight DNA was digested to completion with *Eco*RI, ligated to 10 μg of the isolated *Eco*RI arms of λgtWES · λB (32), and packaged into phage (9).

Recombinant phage (approximately 10^6) were screened without amplification by the method of Benton and Davis (1), using nick-translated insert corresponding to probe 5 (Fig. 1). Positive plaques were purified, and the *Eco*RI inserts were subcloned into the *Eco*RI sites of the plasmid vector pBR322 (3) or pUC8 (37). The resulting recombinant plasmids have been designated pTR1.3 [*Mtv-8*(5′) in pBR322], pTR1.5 [*Mtv-8*(3′) in pUC8], pTR1.10 [*Mtv-9*(3′) in pBR322], and pTR1.1 [*Mtv-17*(3′) in pBR322].

Restriction mapping. Sites of cleavage of restriction endonucleases were mapped by multiple digests of recombinant plasmids. Some sites were mapped with subcloned segments of the relatively large *Eco*RI fragments.

RESULTS

Southern blot analysis of endogenous MMTV genes with site-specific probes. We have characterized the organization of endogenous MMTV sequences in the DNA of several continuous tissue culture cell lines. T1M1 is a T lymphoma of C57BL/6 origin (21, 31); it is unusual among C57BL/6 cells in that it responds to glucocorticoid hormones with synthesis of MMTV RNA (20, 31). VL3 is a T lymphoma derived from the inbred strain C57BL/Ka, a strain closely related to C57BL/6 (16). We have included it in our analysis because it is of the same differentiated cell type as T1M1 but does not express detectable MMTV RNA (D. Peterson, unpublished data). As shown below, our experiments do not reveal any differences between endogenous MMTV DNA in T1M1 and VL3 cells. A BALB/c T lymphoma, W7 (14), has also been included in our experiments because it has been reported that C57BL/6 and BALB/c share sites of endogenous MMTV provirus integration (33).

Most units of MMTV proviral DNA contain single recognition sites for the restriction endonucleases *Eco*RI and *Hin*dIII (4, 6, 13, 15, 25, 33) (Fig. 1). Each such provirus therefore generates a pair of *Eco*RI or *Hin*dIII fragments the sizes of which are determined by the location of these restriction sites in adjacent, nonviral DNA. Each pair of fragments is thus characteristic of a particular site of proviral insertion.

We have characterized the organization of endogenous MMTV sequences by Southern blot analysis of DNA digested with *Eco*RI or *Hin*dIII and probed with nick-translated DNA specific for the various regions of the MMTV genome shown in Fig. 1. Probes 1 and 2 are specific for the 5′ and 3′ (with respect to the direction of MMTV RNA synthesis) *Eco*RI or *Hin*dIII restriction fragments expected from each proviral unit. Probe 3 is specific for LTR sequences, and together probes 4 and 5 represent the entire viral genome.

The sequence organization of MMTV DNA that we have determined is not unique to the T-lymphoma cell lines described above. We have observed the same *Eco*RI or *Hin*dIII restriction fragments in DNA isolated from other C57BL/6 cell lines and from C57BL/6 spleen or liver. Representative results are presented in Fig. 2. These results are particularly significant in view of the findings of Dudley and Risser (8) that many (but not all) T lymphomas of C57BL/6 and BALB/c origin contain amplified MMTV proviral DNA. The cell lines used in the present study, however, contain only the endogenous genes characteristic of the inbred strain of origin.

Southern blots of *Eco*RI-digested DNA are shown in Fig. 3. T1M1 and VL3 DNA generate six *Eco*RI restriction fragments that hybridize to the MMTV probes: three 5′ fragments (10.0, 8.0, and 7.7 kb) and three 3′ fragments (9.7, 8.4, and 6.6 kb). All six hybridize to the LTR probe.

The corresponding blots of *Hin*dIII-digested DNA are shown in Fig. 4, and they reveal seven restriction fragments that hybridize to the MMTV probes: four 5′ fragments (9.1, 8.4, 6.8, and 1.2 kb) and three 3′ fragments (10.0, 7.5, and 4.7

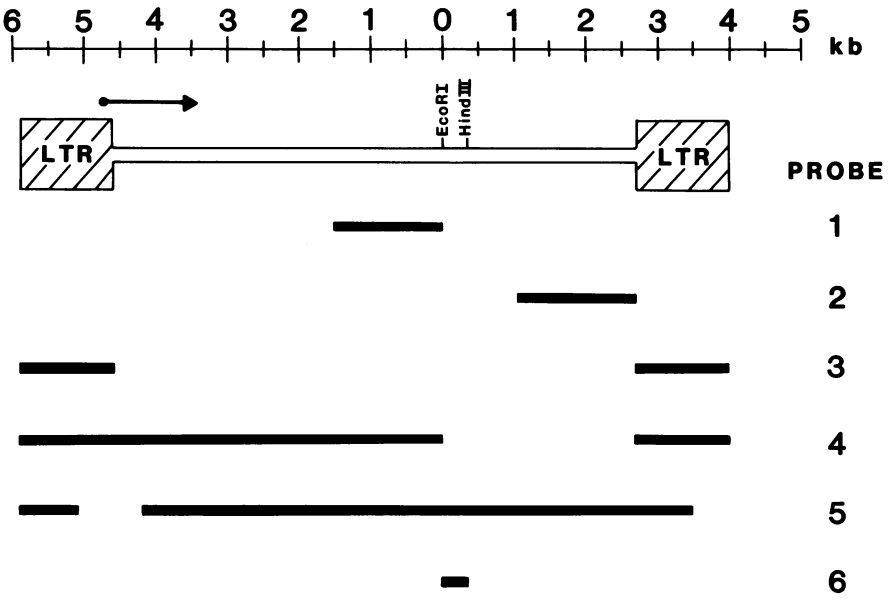

FIG. 1. MMTV hybridization probes. A prototype MMTV provirus is shown, with the LTRs indicated by hatched boxes. The locations of expected *Eco*RI and *Hin*dIII restriction sites are indicated. The cap site and direction of transcription of viral RNA are shown by the arrow. Each of the six probes consists of a recombinant DNA plasmid containing an insert capable of hybridizing to the proviral regions shown. The detailed construction of the plasmids is described in the text.

kb). The six largest fragments hybridize to the LTR probe. The 1.2-kb *Hin*dIII fragment arises from one unit of proviral DNA that contains two *Hin*dIII sites, the expected site (Fig. 1) and a second site located 1.2 kb to the 5′ side. This 1.2-kb fragment is linked to the 6.8-kb 5′ *Hin*dIII fragment. This linkage is demonstrated by the relatively weak hybridization of the 6.8-kb fragment to probe 1 (Fig. 4) and the lack of hybridization of this fragment to probe 6, a probe that hybridizes to the 1.2-kb *Hin*dIII fragment as well as to the 9.1- and 8.4-kb 5′ fragments (data not shown).

In addition to the blots of T1M1 and VL3 DNA, Fig. 3 and 4 also show the MMTV sequences present in the W7 cell line

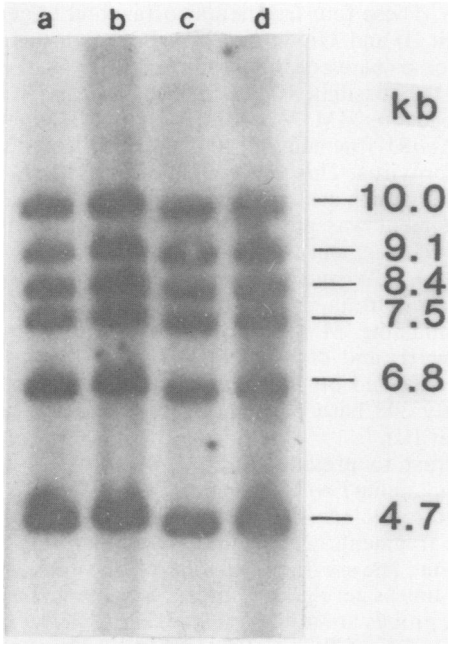

FIG. 2. Comparison of MMTV proviral DNA in T-lymphoma cell lines and that in other C57BL/6 cells. DNA (10 μg) was digested to completion with *Hin*dIII and subjected to Southern blot analysis, utilizing probe 5. Lane a, T1M1 DNA; lane b, VL3 DNA; lane c, 6PG19 (a C57BL/6 melanoma cell line) DNA; lane d, C57BL/6 spleen DNA. The 1.2-kb *Hin*dIII fragment (see Fig. 4) is not shown in this experiment, but it is present in all four of the DNA samples.

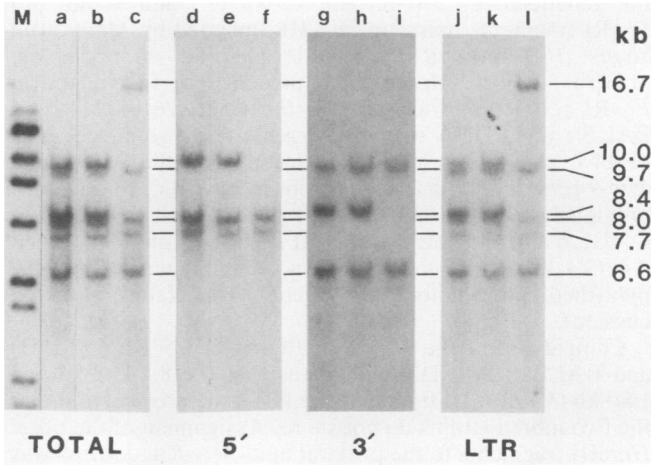

FIG. 3. Southern blots of *Eco*RI-digested DNA. DNA (10 μg) was digested to completion with *Eco*RI and subjected to Southern blot analysis. Lanes a, d, g, j, T1M1 DNA (C57BL/6); lanes b, e, h, k, VL3 DNA (C57BL/Ka); lanes c, f, i, l, W7 DNA (BALB/c); lane M, size markers. Blots were probed with nick-translated DNA hybridizing to total MMTV DNA (mixture of probes 4 and 5; lanes M, a to c), 5′-specific sequences (probe 1; lanes d to f), 3′-specific sequences (probe 2; lanes g to i) and LTR sequences (probe 3; lanes j to l). Lanes a to c and d to f (and also lanes g to i and j to l) represent the same nitrocellulose filter hybridized sequentially with two probes. All DNA samples were subjected to electrophoresis on the same agarose gel.

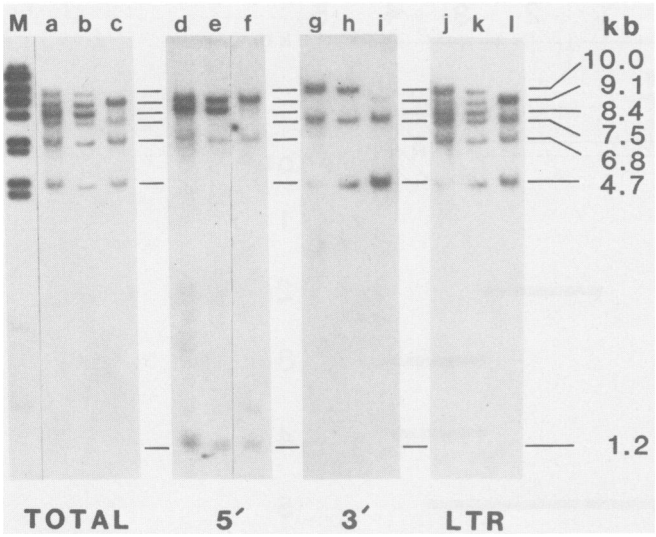

FIG. 4. Southern blots of *Hin*dIII-digested DNA. DNA (10 μg) was digested to completion with *Hin*dIII and subjected to Southern blot analysis. Lanes a, d, g, j, T1M1 DNA (C57BL/6); lanes b, e, h, k, VL3 DNA (C57BL/Ka); lanes c, f, i, l, W7 DNA (BALB/c); lane M, size markers. Blots were probed with nick-translated DNA hybridizing to total MMTV DNA (mixture of probes 4 and 5; lanes M, a to c), 5′- specific sequences (probe 1; lanes d to f), 3′-specific sequences (probe 2; lanes g to i), and LTR sequences (probe 3; lanes j to l). Lanes a to c and d to f (and also lanes g to i and j to l) represent the same nitrocellulose filter hybridized sequentially with two probes. All DNA samples were subjected to electrophoresis on the same agarose gel.

(BALB/c). Endogenous MMTV gene organization in BALB/c cells has been relatively clearly defined (6, 33). The 8.0-kb (5′) and 6.6-kb (3′) *Eco*RI fragments correspond to a single proviral unit that has been termed unit II and is encoded by the genetic locus *Mtv-8*. The 7.7-kb (5′) and 9.7-kb (3′) *Eco*RI fragments make up unit III, encoded by *Mtv-9*. The largest, 16.7-kb *Eco*RI fragment is apparently an incomplete provirus (unit I, *Mtv-6*). The presence of four identical *Eco*RI restriction fragments in the C57BL/6 (T1M1) and BALB/c (W7) DNA strongly suggests that two endogenous MMTV proviruses in C57BL/6 comprise the *Mtv-8* and *Mtv-9* genetic loci that are present in BALB/c, as has been previously reported (33). A third proviral unit is present in C57BL/6 and is defined by *Eco*RI fragments of 10.0 (5′) and 8.4 (3′) kb. This unit is not accommodated within the published endogenous MMTV gene nomenclature (see Discussion).

Comparison of the *Hin*dIII patterns of C57BL/6 (T1M1) and BALB/c (W7) DNA indicates that the 8.4-kb (5′) and 10.0-kb (3′) *Hin*dIII fragments make up the proviral unit that the two inbred strains do not share. Assignment of the other *Hin*dIII fragments to the proviral units *Mtv-8* and *Mtv-9* was made from the restriction maps of our cloned MMTV sequences (see below) and by a double digest of C57BL/6 DNA with *Hin*dIII and *Eco*RI to determine that the 7.7-kb *Eco*RI fragment (5′ portion of *Mtv-9*) contains the *Hin*dIII site responsible for generating the 1.2-kb internal *Hin*dIII fragment (data not shown). The results are summarized in maps given in Fig. 5, which also includes restriction sites mapped from cloned DNA (see below).

Molecular cloning of endogenous MMTV sequences. All six of the *Eco*RI fragments from C57BL/6 that contain MMTV sequences (6.6 to 10.0 kb) are of the appropriate size to lead to viable recombinant bacteriophage when inserted into the vector λgtWES · λB (32). We therefore digested DNA from T1M1 cells to completion with *Eco*RI and ligated it to the isolated arms of this vector. Recombinant molecules were packaged in vitro, and the resulting phage (approximately 10^6 recombinants) were screened without amplification with probe 5 (Fig. 1), a probe homologous to all six of the desired fragments.

With this strategy we isolated recombinant phage with inserts corresponding to four of the six MMTV-containing *Eco*RI fragments: the entire provirus (with 5′ and 3′ flanking DNA) comprising *Mtv-8* (unit II), and the 3′ halves of both *Mtv-9* (unit III) and the endogenous unit present in C57BL/6 DNA but not in BALB/c DNA. We obtained multiple isolates of each of these fragments. The *Eco*RI inserts were subcloned into the *Eco*RI site of the plasmid vector pBR322 (3) or pUC8 (37).

Figure 6 shows a Southern blot in which we directly compare the sizes of the plasmid inserts with the genomic fragments, verifying the identity of the cloned DNA. Each cloned fragment hybridizes with the site-specific probes in Fig. 1 in the expected fashion (data not shown).

We have mapped the sites of cleavage of a number of restriction endonucleases in the cloned DNA. These sites are given in Fig. 5, in which the solid lines represent cloned sequences and the dotted lines represent DNA we have not yet isolated. The positions of the LTRs in the cloned DNA were determined from Southern blots of appropriately digested recombinant plasmid DNA, using a probe specific for the LTR (probe 3, Fig. 1).

DISCUSSION

C57BL/6 DNA contains three MMTV proviral units. Traina et al. (33) have reported that DNA from C57BL/6 mice generates at least four *Eco*RI fragments containing MMTV sequences. These four fragments correspond to genetic loci *Mtv-8* (unit II) and *Mtv-9* (unit III). Based on the results of genetic crosses between different inbred strains, these workers raised the possibility that C57BL/6 DNA might contain a third endogenous MMTV unit (*Mtv-15*, unit X) composed of a single *Eco*RI fragment of 10.0 kb and not containing a complete provirus. This unit could not be assigned to any of the parental strains used in their crosses because the 10.0-kb *Eco*RI fragment was not electrophoretically resolved from the 3′ EcoRI fragment of *Mtv-9* (unit III).

Long et al. (17) have reported that DNA from the closely related inbred strain C57BL/10 generates five *Eco*RI fragments containing MMTV sequences; four comprise the *Mtv-8* (unit II) and *Mtv-9* (unit III) loci, whereas the fifth fragment migrated on their gels with an apparent size approximately 300 base pairs larger than the 3′ fragment of *Mtv-9* (unit III).

In contrast to previous studies, we have observed six MMTV containing *Eco*RI fragments in C57BL/6 DNA. These correspond to the *Mtv-8* and *Mtv-9* loci (see below) and two additional fragments that define what apparently is a third proviral unit. The six fragments that we see are very difficult to resolve unless gel electrophoresis is prolonged or site-specific DNA probes are utilized (Fig. 3). We suspect that in the previous studies of Traina et al. (33) and Long et al. (17) the 10.0-kb (5′) and 8.4-kb (3′) fragments of the third proviral unit were not resolved from the 9.7-kb (3′, *Mtv-9*) and 8.0-kb (5′, *Mtv-8*) fragments with probes representing the entire MMTV genome. Furthermore, the segregation of the third proviral unit as a pair of *Eco*RI fragments (10.0 [5′] and 8.4 [3′] kb) in genetic crosses (33) would have been very difficult

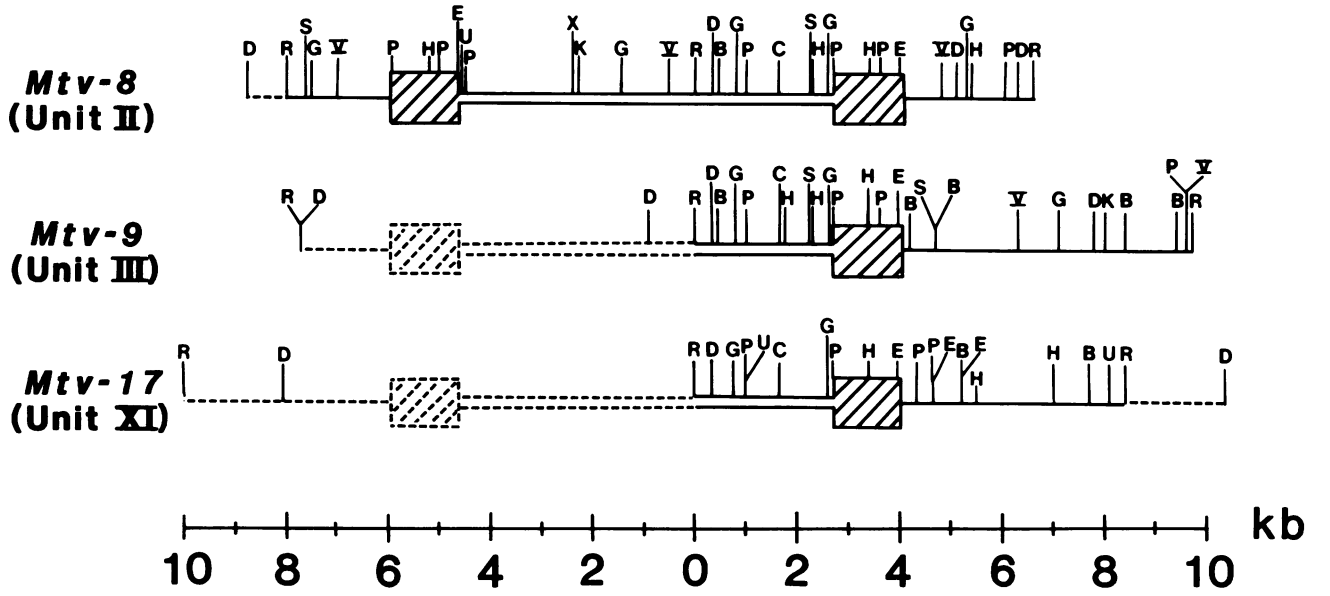

FIG. 5. Restriction maps of MMTV DNA endogenous to C57BL/6 mice. MMTV sequences are shown as double lines; flanking DNA is shown as single lines. LTRs are indicated by hatched boxes. Solid lines represent cloned DNA sequences; dotted lines represent sequences that have not been cloned. We have mapped only *Eco*RI and *Hin*dIII sites in the uncloned DNA. B, *Bam*HI; C, *Cla*I; D, *Hin*dIII; E, *Bst*EII; G, *Bgl*II; H, *Hpa*I; K, *Kpn*I; P, *Pst*I; R, *Eco*RI; S, *Sst*I; U, *Pvu*II; X, *Xho*I; V, *Eco*RV.

to distinguish from the 9.7-kb (3′) and 7.7-kb (5′) fragments of *Mtv-9* unless site-specific probes were used.

The endogenous proviral unit that we have identified appears to be different from any of the 16 endogenous units that have been described previously (33). It is likely that this unit has been confused with *Mtv-9*, which generates *Eco*RI fragments of similar size, but it can be resolved from *Mtv-9* with site-specific probes or by *Hin*dIII digestion of genomic DNA (Fig. 3 and 4). It is clearly different from the incomplete provirus associated with *Mtv-15* (unit X), and we have no evidence that *Mtv-15* is present in C57BL/6 DNA. We tentatively label the endogenous locus we have identified as *Mtv-17* and the proviral unit it defines as unit XI.

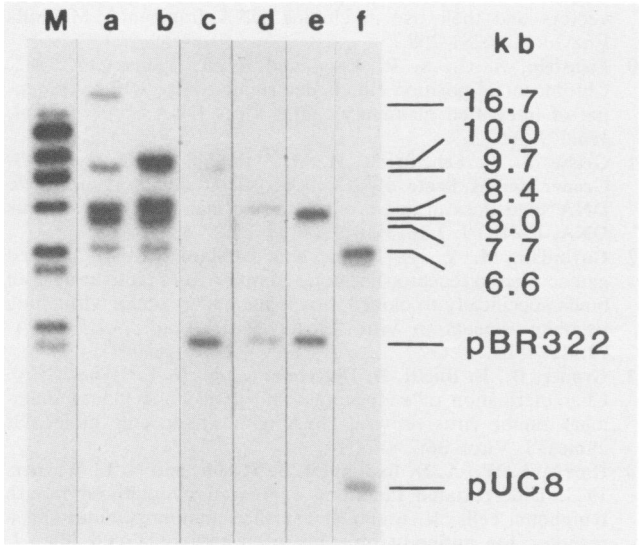

FIG. 6. Sizes of cloned endogenous MMTV DNA fragments. DNA (10 μg of mouse genomic DNA, 100 pg of plasmid DNA) was digested with *Eco*RI and subjected to Southern blot analysis. The blot was probed with nick-translated DNA hybridizing to total MMTV DNA (mixture of probes 4 and 5). Lane M, Size markers; lane a, W7 DNA (BALB/c); lane b, T1M1 DNA (C57BL/6); lane c, plasmid pTR1.10 [*Mtv-9*(3′)]; lane d, plasmid pTR1.1 [*Mtv-17*(3′)]; lane e, plasmid pTR1.3 [*Mtv-8*(5′)]; lane f, plasmid pTR1.5 [*Mtv-8*(3′)].

Sequence organization of endogenous MMTV proviruses. All of our evidence indicates that each of the three endogenous units of MMTV DNA in C57BL/6 has the sequence organization of a complete provirus. For the cloned DNA segments the relative positions of the LTRs with respect to the internal *Eco*RI and *Hin*dIII sites and the hybridization with 5′- and 3′-specific probes clearly demonstrate the appropriate sequence organization. Furthermore, although not identical, the patterns of restriction sites of each of the 3′ fragments are highly conserved in the MMTV portions of the clones, whereas the 3′ flanking regions are variable. The restriction maps of the viral genomes of all of the cloned fragments are very similar to published maps of proviral DNA that codes for infectious MMTV virions (4, 25). For the two uncloned 5′ fragments the sequence organization cannot be as clearly defined. However, the specific hybridization of these fragments to 5′ and LTR-specific probes (Fig. 3 and 4) is consistent with the expected proviral sequence organization.

The restriction sites we have mapped in *Mtv-8* and *Mtv-9* are almost identical to those reported for these loci in BALB/c mice obtained by mapping genomic DNA (6). This identity extends throughout the MMTV proviruses and flanking DNA. Despite the lack of a documented common lineage (30), this result strongly supports the notion that these proviruses are inserted at identical sites in the two inbred strains. The restriction map of the *Mtv-8* locus that we have determined is also essentially identical to that of the analogous locus cloned from GR mice (15).

The failure to recover two of the 5′ *Eco*RI fragments in our cloning strategy occurred despite the screening of approximately 10^6 recombinant phage (which we estimate to have

given us a 95% probability of recovering all six MMTV-containing fragments). Similar observations concerning the relative difficulty of cloning 5′ segments of MMTV proviral DNA have been reported by others (13, 18, 34). There appears to be a "poison" region approximately 1 kb in size that for unknown reasons is propagated very poorly in *E. coli*. We are currently using other cloning strategies to clone at least portions of the 5′ fragments we have been unable to isolate.

Expression of endogenous MMTV genes. Our analysis of the endogenous MMTV sequences in C57BL/6 DNA has failed to reveal any obvious deletions or rearrangements that would render them defective. In fact, several lines of evidence suggest that one or more of these endogenous proviral elements contains all of the sequence information necessary to specify hormone-mediated transcription. The C57BL/6-derived cell line T1M1, from which we have cloned the endogenous MMTV sequences reported here, responds to glucocorticoid hormones with synthesis of MMTV RNA (20, 31). At the level of our blot analysis, the endogenous proviral units in T1M1 are identical to those of other cells in which they are not expressed (e.g., VL3, Fig. 3 and 4). In addition, the endogenous provirus present at the *Mtv-8* locus and isolated from GR mice is fully capable of specifying glucocorticoid-regulated transcription after transfection into tissue culture cells (15). We have obtained similar results with our clone of *Mtv-8* DNA (P. Burdick and D. Peterson, unpublished data).

In the absence of demonstrable differences in the proviral DNA itself, it is possible that cell-specific differences in endogenous MMTV gene expression could be determined by the presence or absence of *trans*-acting factors that interact with regulatory sequences on the proviral DNA (e.g., the glucocorticoid receptor protein). The evidence, however, suggests that this is not the case. Several C57BL/6 or BALB/c cell lines that do not express endogenous MMTV genes have biochemically identifiable glucocorticoid receptor proteins and can hormonally regulate exogenous MMTV genes acquired by virion infection (31; D. Peterson, manuscript in preparation). Thus, even though these cells do not express endogenous MMTV genes, they appear to contain all *trans*-acting factors necessary to interact with the viral genome and activate synthesis of MMTV RNA.

Another possibility is that determination of activity (or inactivity) of endogenous MMTV genes may be indirectly controlled by sequences flanking the provirus itself. Such position effects could be mediated by factors that establish or maintain permissive chromatin domains at the appropriate genetic loci. Consistent with this notion is the observation that at least one endogenous proviral unit in T1M1 cells (but not in other cells in which MMTV RNA is not synthesized) is marked by a site of nuclease hypersensitivity that maps to the glucocorticoid response element in the 5′ LTR of the provirus (D. Peterson, Mol. Cell. Biol., in press). Such hypersensitive sites are also a characteristic of exogenously acquired MMTV genomes in cells that hormonally regulate the synthesis of viral RNA (Peterson, unpublished data).

Little is known about the molecular mechanisms involved in the developmental commitment of steroid-responsive genes, that is, the molecular signals that program certain genes to be hormone responsive in one differentiated cell type but not in another. Analysis of endogenous MMTV genes that are normally not transcriptionally active and the definition of conditions under which they become hormone responsive may help reveal aspects of the molecular mechanisms by which this developmental commitment occurs.

ACKNOWLEDGMENTS

We are grateful to David Bieber and Stuart Feinstein for providing valuable help in the early stages of this work. We also thank Polly Boren for help in constructing restriction maps and Paige Franks for expert preparation of the manuscript.

This work was supported by Public Health Service grant CA32695 from the National Cancer Institute and grant A-904 from the Robert A. Welch Foundation. This work was also partially funded by the Texas Agricultural Experiment Station.

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